

Death-Associated Protein Kinase Loss of Expression Is a New Marker for Breast Cancer Prognosis

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ABSTRACT

Purpose: Death-associated protein (DAP)-kinase is a new Ser/Thr kinase involved in cell apoptosis and tumor suppression, the expression of which has been correlated to invasive potential and metastasis in several human neoplastic tissues. We analyzed the level of DAP-kinase expression in breast cancer specimens and its correlation with survival.

Experimental Design: One hundred twenty-eight breast cancer specimens were analyzed by immunohistochemistry. Patient records were studied retrospectively for demographic characteristics, clinical data, hormonal treatment, outcome, and survival. DAP-kinase protein expression was also studied in normal breast cells primary cultures under estrogen and antiestrogen treatment.

Results: Among the 128 patients, 30 showed a DAP-kinase staining $\leq 20\%$, whereas 98 had a staining over 20%. Mean follow-up time was 62 months. The association between tumor Scarff-Bloom and Richardson grade ($P = 0.009$), estrogen receptor and progesterone receptor expression ($P = 0.002$ and 0.001 , respectively), tumor size ($P = 0.05$), Bcl-2 expression ($P = 0.004$), and DAP-kinase immunostaining in the ductal carcinoma group was highly significant. Overall (64 months) and disease-free (63 months) survival in the high DAP-kinase expression group were significantly longer compared with the women whose tumors showed a loss of DAP-kinase expression (51 and 43 months, respectively). DAP-kinase protein was strongly expressed in normal breast tissue and in human breast epithelial cells primary cultures. Estradiol decreased DAP-kinase expression in these cells, arguing for hormonal regulation of the protein.

Conclusions: Loss of DAP-kinase expression negatively correlates to survival and positively correlates to the prob-

ability of recurrence in a very significant manner. DAP-kinase thus constitutes a novel and independent prognosis marker for breast cancer.

INTRODUCTION

Breast cancer is one of the most common causes of cancer-related deaths in women. Despite improvements in diagnosis and treatment of this disease in the past decades, the survival rates remain low in comparison with other cancers (1). With advances in detection means of breast cancer, more patients are now diagnosed at earlier stages of the disease. However, it currently remains difficult to foresee whether those patients will be cured with surgery alone or should benefit from additional and more aggressive treatments to improve their long-term survival. It is therefore important that new, clinically relevant, and easily measurable markers be validated in breast tumors with the aim of improving initial staging and allowing a patient-to-patient therapeutic management. Patients with initial higher risk of recurrence or metastasis could benefit from appropriate adjuvant therapeutics in addition to complete surgical resection of the primary tumor.

Inactivation of tumor suppressor genes and decreased cell apoptosis are essential mechanisms for breast tumorigenesis, contributing to deregulated tumor cell proliferation, invasion, and metastasis (2). Tumor sensitivity to any given therapeutic regimen is commonly mediated by initiation of programmed cell death via available active apoptotic pathways (3). Several genes belonging to a defined apoptotic cascades such as p53 have already demonstrated their association with breast tumor prognosis (4).

Death-associated protein (DAP)-kinase is a novel multidomain calcium/calmodulin-regulated and cytoskeletal-associated serine/threonine kinase mandatory for IFN- γ , tumor necrosis factor α , and activated Fas-induced apoptotic cell death and detachment from the extracellular matrix, comprising modules such as ankyrin repeats mediating protein-to-protein interactions as well as a death domain (5). This 160 kDa protein kinase is normally localized in the cytoskeleton in association with actin microfilaments. The death-promoting effects of DAP-kinase depend on its intact catalytic activity, the correct intracellular localization, and the presence of the death domain (6). The relation between DAP-kinase and human cancer has already been suggested. It was found that DAP-kinase mRNA and protein expression are frequently lost in various human cancer cell lines such as B-cell lymphoma and leukemia cell lines, as well as bladder, breast, and renal cell carcinoma-derived cell lines, highlighting its potential role as a tumor suppressor gene (7, 8). Moreover, loss of DAP-kinase expression was shown to correlate strongly with recurrence and metastasis incidence and thus with pejorative prognosis of several human cancers such as small cell lung cancer, B-cell malignancies, primary head and neck tumors, colon and bladder cancers, and multiple myeloma

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(6, 9). Finally, restoration of DAP-kinase expression to physiological levels in a murine model of highly metastatic lung carcinoma cells can strongly suppress their metastatic ability (10). It appears that loss of DAP-kinase expression confers a selective advantage to cancer cells and may play a causative role in tumor progression. Thus, DAP-kinase expression may be useful in identifying aggressive tumors more likely to spread and alter patients long-term survival. Because no data have yet been published on DAP-kinase protein expression in breast cancer, we addressed this question by studying DAP-kinase expression on breast tumor specimen using immunohistological staining. In addition, we took advantage of cultures of normal breast cells routinely developed in our laboratory to study protein expression and hormonal regulation.

PATIENTS AND METHODS

Study Population. A total of 128 patients who had been diagnosed with breast cancer and undergone tumorectomy or mastectomy for complete resection of their primary tumors at the Hôtel Dieu Hospital of Paris Gynecology Department, during the period from June 1985 through May 1998, was included in the study. Patients eventually received adjunct chemotherapy and/or radiotherapy according to initial staging. Fifty-seven postmenopausal patients with invasive ductal carcinoma staining positively for estrogen receptor (ER) received postoperative hormone therapy with tamoxifen for a mean period of 5 years. Patients were followed-up by the same physician (Y. Decroix) for a mean follow-up time of 66 months. Survival and follow-up durations were measured as the time between the first oncology consult after treatment completion and the last consult in the department or death. Patient records were reviewed retrospectively for demographic characteristics, clinical data, outcome, and survival. Tissue sections were obtained from each tissue block for immunohistochemistry staining, whereas precise histological diagnosis was confirmed for each case by the same pathologist (D. Hugol).

Immunohistochemistry. The 128 breast tumors were analyzed by immunohistochemistry for DAP-kinase, ER, progesterone receptor (PR), and Bcl-2 staining, using an anti-DAP-kinase monoclonal antibody as previously described (5, 7), an anti-ER- α monoclonal antibody (TEBU, Santa Cruz Biotechnology; mouse monoclonal antibody, Le Perray en Yvelines, France), an anti-PR monoclonal antibody (TEBU, Santa Cruz Biotechnology, mouse monoclonal antibody, Le Perray en Yvelines, France), and an anti-Bcl-2 monoclonal antibody (Dako Cytomation S. A., Trappes, France) on paraffin tumor blocks. Briefly, 8- μ m paraffin-fixed tissue sections were deparaffined and rehydrated. To permeate the cells, slides were immersed in citrate buffer 10 mM (pH 6) and microwaved three times for 5 min. After rinsing thoroughly with Tris-buffered saline (TBS; containing casein and Tween), cells were stained with the antibodies against DAP-kinase, ER- α , PR, and Bcl-2 (dilution 1/200, 1/100, 1/50, and 1/50, respectively) for 2 h at room temperature. Slides were washed three times with TBS for 5 min, then incubated with the biotinyl goat antimouse immunoglobulin coupled to biotin (dilution 1/200) for 20 min (Valbiottech, Dynal, France), followed by three TBS washes. The streptavidin biotin peroxidase complex was applied in TBS

(dilution 1/200) to the sections for 45 min, followed by three TBS washes. Slides were flooded with 8 mg of 3,3'-diaminobenzidine hydrogen peroxide chromogen in the dark for 5 min and additionally rinsed with distilled water. Finally, slides were immersed in hematin for 30 s and mounted for light microscopy analysis. The resultant staining was evaluated both for DAP-kinase and Bcl-2 by determination of the percentage of stained invasive tumor cells on a given paraffin slide. Staining for DAP-kinase and Bcl-2 were achieved on two serial sections belonging to the same paraffin block.

Human Breast Epithelial (HBE) Cells Cultures. Breast tissue was obtained from 5 women in ages between 15 and 25 years undergoing reduction mammoplasty. The patients had no history of breast disease and pathological analysis showed only normal breast tissue. Sampling of the tissue was performed according to the French governmental regulations on clinical experimentation. The epithelial cells were plated and grown as described previously (11). Briefly, the tissue was digested with 0.15% collagenase (Roche Diagnostics, Meylan, France) and 0.05% hyaluronidase (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France) in Ham's F10 medium (Invitrogen) and filtered through 150- μ m sieves to retain undigested tissue. Cells were grown in Ham's F10 medium with phenol red, supplemented with 0.24% NaHCO₃ (Invitrogen), 1% penicillin (10,000 units)/streptomycin (10 mg; Sigma), 5 ng/ml cortisol (Sigma), 6.5 ng/ml triiodothyronine (Sigma), 10 ng/ml cholera toxin (Sigma), 5 mg/ml transferrin (Sigma), 5% compatible human serum, 0.12 units/ml insulin (Sigma), and 10 ng/ml epidermal growth factor (Sigma) in a humidified atmosphere of 5% CO₂, 95% air. For comparison in the level of DAP-K expression, breast cancer cell lines were also grown. MCF-7, T47-D, and ZR75-1 cell lines were a gift from Dr. Christine. Mercier-Bodard (Kremlin-Bicêtre, France) and originally came from the laboratories of Dr. Marc Lippman (Bethesda, MD) and Dr. Kate Horwitz (Denver, CO). MCF-7, ZR75-1, and MDA-MB-231 cells were grown in DMEM (Invitrogen, France) without phenol-red, supplemented with 5% FCS and 2 mM glutamine, T-47-D in RPMI 1640 supplemented with 5% FCS and 2 mM glutamine (Invitrogen).

Hormonal Treatments. Before hormonal treatments, HBE cells were synchronized in a medium containing 20 μ M lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, for 40 h in Ham's F-10 without phenol red, and synchronization was stopped by adding 2 mM mevalonate to the hormone-containing medium (12). Subsequently, cells were treated 48 h in a phenol red free medium containing 5% of compatible human serum with 10 nM estradiol (E₂) and 1 μ M of a potent steroidal antiestrogen RU58668 (RU58).

Western Immunoblotting. DAP-kinase protein expression was measured in cultured normal human epithelial breast cells by Western blot technique as previously described (13) and modified as following. Extraction of the proteins was performed using a buffer containing DOC (0.5%), SDS (0.1%), NP40 (1%), and two antiproteases, 100 μ g/ml phenylmethylsulfonyl fluoride (Boehringer) and 1 μ g/ml aprotinin (Sigma-Aldrich). The extracted proteins were measured using BCA (Pierce, Interchim, Montluçon, France). Eighty μ g of protein from HBE cells were analyzed by 8% SDS-PAGE. DAP-kinase immunoreactivity was detected using a mouse

Table 1 Patient clinical characteristics stratified by tumor histological type

	Histological Type [n patients (%)]		<i>P</i> ^a
	Ductal	Lobular	
	<i>n</i> = 105 (82%)	<i>n</i> = 23 (18%)	
Age in years [mean (SE)]	56.3 ± 1.3	53.5 ± 2.3	NS ^b
Body mass index [mean (SE)]	23.9 ± 0.4	23.4 ± 0.7	NS
Menopausal status			
Patient no. [n (% of patients)]	65 (62%)	14 (61%)	
Age at menopause in years [mean (SE)]	52.0 ± 0.3	51 ± 0.7	NS
Hormone replacement therapy (ever users)			
Patient no. [n (% of patients)]	17 (26%)	8 (57%)	NS
Hormone replacement therapy, duration in months [mean (SE)]	58.4 ± 15.2	46.5 ± 14.1	NS
Parity			
No of children [mean (SE)]	1.6 ± 0.2	1.8 ± 0.3	NS
Breast feeding			
Patients no. [n (% of patients)]	36/77 (47%)	10/20 (50%)	NS
Cumulative duration in months [mean (SE)]	6.3 ± 2.1	4.2 ± 0.9	NS
Benign breast disease ^c			
Patients no. [n (% of patients)]	21 (20%)	5 (22%)	NS
Current smoking [n (% of patients)]	22/85 (26%)	8/23 (35%)	NS
Tumor size (cm) [mean ± SE]	2.0 ± 0.1	1.5 ± 0.2	0.07
Invaded nodes [n number of cases studied]	38/101 (18%)	10/21 (48%)	NS
Disease-specific deaths during follow-up	17	1	0.13
5-years survival probability (95% confidence interval)			
Overall (deaths)	17 (17%)	3 (13%)	NS
Disease-specific (recurrence)	17 (17%)	1 (4%)	0.13
Disease-free survival in months	57.4 ± 3.7	65.9 ± 9.0	NS

^a Statistical significance is defined as *P* < 0.05.

^b NS, nonsignificant.

^c Benign breast disease is considered as present when breast examination, mammogram, and breast sonogram coincide with the diagnosis.

monoclonal antibody at 1:2500 provided by Kimchi *et al.* (5, 7). Relative amounts of proteins were quantified by scanning densitometry using the software program RAG (Biocom, Les Ulis, France).

Statistics. Association between the level of DAP-kinase expression and the various prognosis markers was analyzed with

SPSS and BMDP softwares using χ^2 and Student *t* tests. Correlation with disease-free survival time was performed by Kaplan-Maier and Cox model survival analysis. Nonparametric Mann-Whitney tests were used to compare the effects of hormonal treatments in HBE cells. One-way ANOVA test and multiple range Student-Newman-Keuls tests were performed to

Table 2 Outcome data and prognosis markers stratified by DAP-kinase expression level in the 105 invasive ductal carcinoma

Death-associated protein kinase-positive invasive tumor cells [%]	≤ 20%	> 20%	<i>P</i> ^a
	<i>n</i> = 28	<i>n</i> = 77	
Age in years [mean (SE)]	55.4 ± 3.3	56.6 ± 1.4	NS ^b
Menopausal cases [n (% of patients)]	15/28	50/77	NS
Hormone replacement therapy (ever users, <i>n</i> patients/ <i>N</i> cases studied)	2/27	15/74	NS
Tumor size in cm [mean (SE)]	2.3 ± 0.3	1.8 ± 0.1	0.05
Invaded nodes (<i>n</i> / <i>N</i> cases studied)	12/26	26/75	NS
Positive estrogen receptor (<i>n</i> patients/ <i>N</i> cases studied)	14/28	61/73	0.002
Positive progesterone receptor [n patients (% of patients)]	13/28	62/72	0.001
Scarff Bloom and Richardson prognosis score grade:			
1	7	31	
2	10	35	
3	10	8	0.009
(<i>n</i> = 101 studied cases)	<i>n</i> = 27	<i>n</i> = 74	
Tamoxifen use during follow-up	7/28	50/77	0.0006
Bcl-2 [% of marked invading tumor cells ± SE;	43.9 ± 6.9	69.5 ± 4.4	0.004
<i>n</i> = 74 cases studied [mean ± SE]	<i>n</i> = 19	<i>n</i> = 55	
Metastasis during follow-up (<i>n</i> / <i>N</i> cases studied)	5	6	0.14
Recurrence during follow-up (<i>n</i> patients/ <i>N</i> cases studied)	11	8	0.004
Deaths during follow-up	10	8	0.006

^a Statistical significance is defined as a *P* < 0.05.

^b NS, nonsignificant.

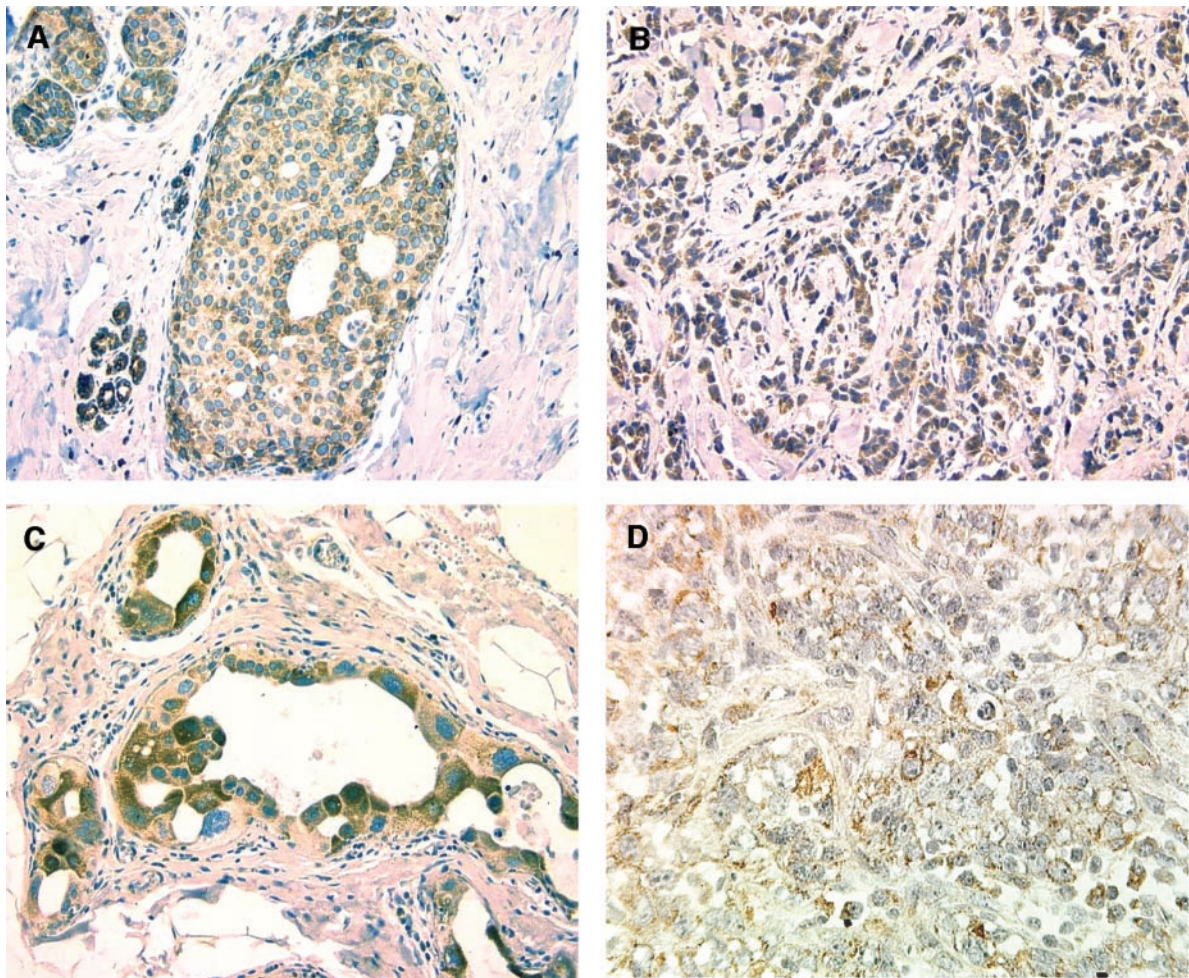


Fig. 1 Immunohistochemical staining for death-associated protein (DAP)-kinase on breast tumor sections, using an anti-DAP-kinase monoclonal antibody. **A**, normal breast tissue and intraductal carcinoma expressing a high level of DAP-K (>80% of the cells are stained), magnification, $\times 20$; **B**, intraductal carcinoma showing heterogeneous staining for DAP-K, $\times 40$; **C**, lobular carcinoma, 80–100% of the cells are stained, $\times 20$; **D**, invasive ductal carcinoma showing a significant loss of DAP-K expression, $\times 20$.

compare the relative efficiency of each treatment as was previously described (2).

RESULTS

A total of 105 invasive breast ductal carcinoma and 23 lobular carcinoma specimens was included. Patients' mean age was 55.8 ± 1.3 years (range, 29–89 years) at the time of diagnosis. Twenty patients died during follow-up (mean disease-free survival time, 59 months), whereas 108 patients were still alive after a mean follow-up time of 66 months (mean disease-free survival time, 64 months). The mean overall survival time was 62 months. Among the 20 patients who died, 15 died from breast cancer, 3 of heart disease, and 2 of unknown cause. The general clinical characteristics of the patients, stratified by histological type, are shown in Table 1.

Tumor size, node invasion status, histological Scarff-Bloom and Richardson (SBR) grading, estrogen and progesterone receptor expression, and Bcl-2 expression data in the 105 invasive ductal carcinoma specimens are presented in Table 2.

Among the 105 patients, 28 showed a DAP-kinase staining $\leq 20\%$, 77 had a staining over 20%, whereas $>80\%$ of adjacent normal breast cells showed intense DAP-kinase staining in all of the cases where normal cells were present and staining could be quantified (Fig. 1A–D). DAP-kinase loss of expression was mainly observed in SBR grade 3 and ER-negative tumors, whereas grade 1 and ER-positive tumors displayed high expression levels. More specifically, tumor SBR grade ($P = 0.009$), ER ($P = 0.002$) and PR ($P = 0.001$) expression, tumor size ($P = 0.05$), and Bcl-2 ($P = 0.004$) expression were significantly associated with the DAP-kinase immunostaining level (Table 2). Thus, DAP-kinase expression follows closely the other well-recognized breast cancer prognosis markers. When Kaplan-Maier analysis was performed, stratified on histological type, overall (64 months) and disease-free (63 months) survival in the high DAP-kinase expression group was significantly longer compared with the women whose tumors showed a loss of DAP-kinase expression (51 and 43 months, respectively; Fig. 2). The higher DAP-kinase staining, the better was the overall

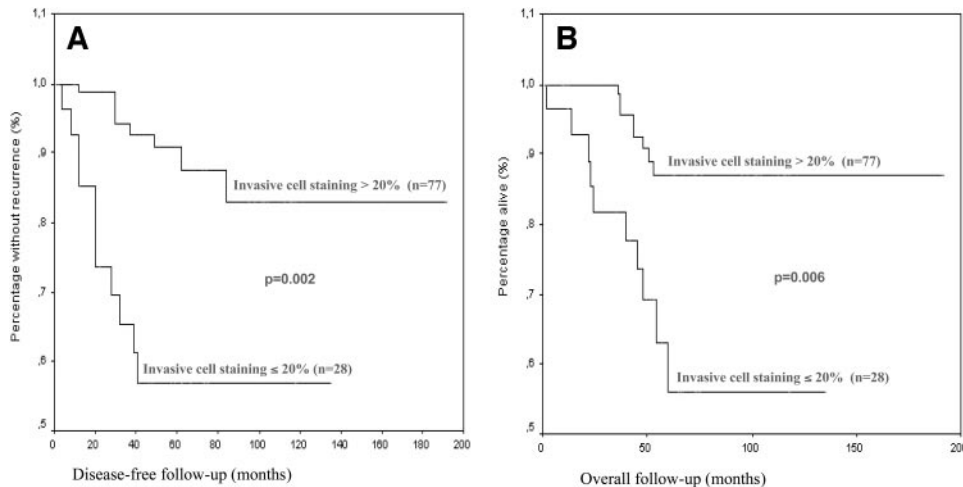


Fig. 2 Kaplan-Meier analysis for disease-free and global survival duration in both low ($\leq 20\%$) and high ($> 20\%$) death-associated protein (DAP)-kinase expression groups of the 105 patients with invasive ductal breast carcinoma. A, probability of disease-free survival for patients with high DAP-kinase expression ($n = 77$) versus patients with low DAP-kinase expression ($n = 28$). B, probability of overall survival for patients with high DAP-kinase expression ($n = 77$) versus patients with low DAP-kinase expression ($n = 28$).

survival prognosis ($P < 0.005$). This result remained valid after adjusting for age, tumor size, ER positivity, and node involvement in the 96 cases studied (data not shown). Finally, using a Cox model analysis, DAP-kinase staining remained an independent prognosis marker after adjustment for SBR grade, ER and PR expression, tumor size, tamoxifen use, and *BclII* expression. Among the 57 tamoxifen-treated patients with ductal carcinoma, 50 had a DAP-kinase staining over 20%, a disease-free and overall survival of 64 and 66 months, respectively, and 22% were node positive. The tamoxifen-treated patients ($n = 7$) exhibiting a low DAP-kinase staining had a significantly lower disease-free (55 months) and overall (62 months) survival ($P = 0.006$), and 43% were node positive. Thus, it appears that

hormonal adjuvant therapy does not modify prognosis association to DAP-kinase level of expression.

Because the DAP-kinase expression was consistently high in the normal breast tissue adjacent to the tumor cells and because its expression was strongly correlated to ER positivity, we used primary cultures of normal human breast cells (HBE cells) developed routinely in our laboratory to study E_2 regulation of DAP-kinase expression in normal breast cells (2). We have previously reported that the normal HBE cultures still express ER and PR under our experimental conditions and thus remain responsive to hormones and antihormones for proliferation and apoptosis measures (11, 14). As shown in Fig. 3 and as expected, DAP-kinase appeared as a single band of 160 kDa in the HBE cell cultures. In contrast, DAP-kinase was not detected in the three studied hormone-dependent cell lines (MCF-7, T47-D, and ZR75-1 cells). A very weak expression was detected in the ER- α -negative cell line, MDA-MB-231 (data not shown). In addition, the DAP-kinase protein level of expression was modulated by E_2 in the HBE cells. A constant, moderate but significant decrease in DAP-kinase expression was observed under cellular E_2 treatment ($P < 0.05$; Fig. 3). This effect was reversed by a potent antiestrogen arguing for an ER-mediated effect ($E_2 + RU58$, NS compared with control and $P < 0.05$ compared with E_2).

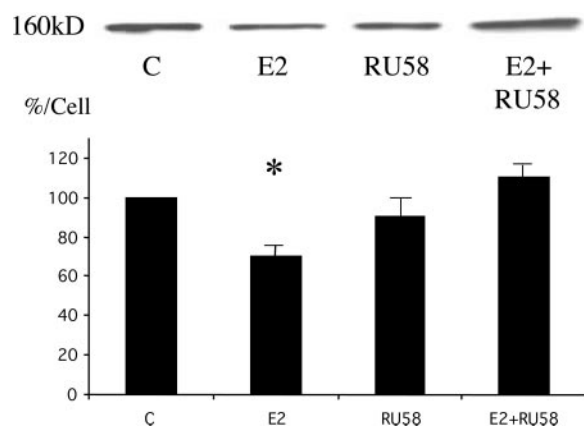


Fig. 3 Death-associated protein (DAP)-kinase protein levels in human breast epithelial cells under estradiol (E_2) and antiestrogen treatments. Human breast epithelial cells were treated for 48 h with 10 nM E_2 alone or with 1 μ M RU 58668. DAP-kinase protein content was estimated by Western blot. The relative amounts of proteins are quantified by scanning densitometry using the software program RAG (Biocom). A representative Western blot is shown above the graph. Data are expressed as percent compared with control. Values are expressed as the mean \pm SE of four separate experiments. *, $P < 0.05$ compared with control and $E_2 + RU$.

DISCUSSION

To our knowledge, this is the first time DAP-kinase level of protein expression is correlated to breast cancer prognosis and survival. We already postulated that loss of DAP-kinase expression *in vitro* may confer a selective advantage during the multiple stages of tumorigenesis and metastasis for tumor cells associated with resistance to various apoptotic stimuli (IFN- γ , tumor necrosis factor α , Fas, detachment from extracellular matrix, oncogenes; Ref. 10). In the present series, the loss of DAP-kinase protein expression very strongly correlated with disease recurrence and disease-free survival duration. At 5 years of follow-up, the overall survival rate was significantly higher in the group expressing high DAP-kinase levels compared with the low expression group, 0.88 and 0.57, respectively (Fig. 2). This

observation remained valid when adjusted for other prognosis markers (tumor size, ER expression, SBR grade, and Bcl-2 expression). The 20% cutoff level of DAP-kinase staining was the first one to demonstrate a significantly different clinical outcome. When groups were split with 25, 30, and 35% invasive cell staining, the difference remained significant.

The decrease in DAP-kinase protein expression observed in cultured normal breast cells seems to be the consequence of a direct E2 effect, as suggested by antiestrogen reversion. This demonstrates that DAP-kinase is yet another target for E2 in apoptosis regulation. We recently reported that E2 was able to alter the cellular expression levels of Bcl-2, p53, and caspase-3 in HBE cells; this effect being also reversed by an antiestrogen agent (15). However, stratifying the data by antiestrogen adjuvant treatment did not significantly modify the prognosis association to DAP-kinase level of expression. This result warrants confirmation because the group of patients having received tamoxifen and exhibiting low levels of DAP-kinase expression was very small ($n = 7$). In addition, E₂ is probably not responsible alone for DAP-kinase regulation because only the ER- α -negative cells (MDA-MB-361) expressed DAP-kinase, whereas the ER- α -expressing cell lines remained negative as reported previously (8). The loss of expression of DAP-kinase in the three cell lines may be related to their specific tumoral phenotype. A recently published study showed that the DAP-kinase promoter was highly methylated in MCF-7 cells, whereas methylation was absent in MDA-MB-231 cells. The degree of promoter methylation, as an epigenetic regulation, could be partly responsible for the variable level of DAP-kinase protein expression in breast tissue. This phenomenon seems to be a gene and histological type-specific one (16).

It was recently reported that a fraction of lobular invasive breast carcinoma exhibited a high level of DAP-kinase loss of expression (mRNA) and promoter hypermethylation (16). DAP kinase inactivation also correlated with ER positivity and the absence of p53 expression. However, this work did not show a correlation between DAP-kinase promoter methylation and DAP-kinase protein level, tumor size, grade or node involvement, and patient survival was not studied. Interestingly, they also described a very low level of promoter methylation in normal breast tissue. Only 9% of their 85 invasive ductal carcinoma specimens showed promoter hypermethylation. In our series, loss of DAP-kinase expression clearly predominated in invasive ductal specimens, whereas only 2 of the 23 lobular tumors exhibited low DAP-kinase expression (<20% of stained cells). It is thus likely that promoter epigenetic hypermethylation is only one possible way of inactivating a gene at the protein level. Others have reported promoter homozygous deletions of CpG island inducing DAP-kinase loss of expression in invasive pituitary tumors (17).

Very little is known about the mechanisms by which DAP-kinase achieves its proapoptotic function. A recent study demonstrated that DAP-kinase activates a p53-mediated pathway (18). DAP-kinase was also described as a negative regulator of integrin activity and cell adhesion, diminishing integrin-mediated survival signals, and that integrin activation blocked DAP-kinase-induced up-regulation of p53 (19). Thus, the DAP-kinase proapoptotic action seems to locate early in the p53-dependent apoptotic pathway; thereby explaining the fact that DAP-kinase

inactivation and p53 overexpression are mutually exclusive (17).

In a recent study, a shorter 5-year survival rate was observed in patients with non-small cell lung cancer with DAP-kinase promoter CpG region hypermethylation (20). DAP-kinase was also shown to be commonly inactivated by promoter hypermethylation in B-cell malignancies (8, 21) and multiple myeloma (9). High fatality and poor treatment outcomes in multiple myeloma are linked to dissemination forms and uncontrolled circulatory pool of multiple myeloma precursors. It is possible that the cellular selective advantage conferred by DAP-kinase inactivation may play a role in the induction and maintenance of circulatory tumor pool. Hypermethylation studies are currently underway in our department to identify the mechanism by which DAP-kinase expression is lost in human breast tumors.

Whether poor prognosis for patients expressing low levels of DAP-kinase protein is directly related to local enhanced tumor cell survival, invasiveness potential, and metastasis proneness remains to be established. Whether DAP-kinase loss of expression can also be observed in a subset of very early-stage breast tumors or in certain types of benign atypical hyperplasia may be important for appropriate management of benign breast disease and *in situ* breast cancer.

In conclusion, in a series of 128 patients followed-up for 60 months, we show that DAP-kinase protein expression constitutes a new and strong independent predictor of breast cancer prognosis and survival.

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REFERENCES

1. Wingo PA, Cardinez CJ, Landis SH, et al. Long-term trends in cancer mortality in the United States, 1930–1998. *Cancer* 2003;97:3133–275.
2. Gompel A, Somaï S, Chaouat M, et al. Hormonal regulation of apoptosis in breast cells. *Steroids* 2000;65:593–8.
3. Darzynkiewicz Z. Apoptosis in antitumor strategies: modulation of cell cycle or differentiation. *J Cell Biochem* 1995;58:151–9.
4. Falette N, Paperin MP, Treilleux I, et al. Prognostic value of p53 gene mutations in a large series of node-negative breast cancer patients. *Cancer Res* 1998;58:1451–5.
5. Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the γ interferon-induced cell death. *Genes Dev* 1995;9:15–30.
6. Cohen O, Kimchi A. DAP-kinase: from functional gene cloning to establishment of its role in apoptosis and cancer. *Cell Death Differ* 2001;8:6–15.
7. Cohen O, Feinstein E, Kimchi A. DAP-kinase is a Ca²⁺/calmodulin-dependant, cytoskeletal associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J* 1997;16:998–1008.
8. Kissil JF, Feinstein E, Cohen O, et al. DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. *Oncogene* 1997;15:403–7.
9. Ng MHL, To KW, Lo KW, et al. Frequent death-associated protein kinase promoter hypermethylation in multiple myeloma. *Clin Cancer Res* 2001;7:1724–9.

10. Inbal B, Cohen O, Polak-Charcon S, et al. DAP kinase links the control of apoptosis to metastasis. *Nature (Lond)* 1997;390:180–4.
11. Gompel A, Malet C, Spritzer P, et al. Progestin effect on cell proliferation and 17 β -hydroxysteroid dehydrogenase activity in normal human breast cells in culture. *J Clin Endocrinol Metab* 1986;63:1174–80.
12. Keyomarsi K, Sandoval L, Band V, Pardee AB. Synchronization of tumor and normal cells from G₁ to multiple cell cycles by lovastatin. *Cancer Res* 1991;51:3602–9.
13. Kandouz M, Siromachkova M, Jacob D, et al. Antagonism between estradiol and progestin on Bcl-2 expression in breast cancer cells. *Int J Cancer* 1996;68:120–5.
14. Malet C, Gompel A, Yaneva H, et al. Estradiol and progesterone receptors in cultured normal human breast epithelial cells and fibroblasts: immunocytochemical studies. *J Clin Endocrinol Metab* 1991;73:8–17.
15. Somaï S, Chaouat M, Jacob D, et al. Antiestrogens are pro-apoptotic in normal human breast epithelial cells. *Int J Cancer* 2003;105:607–12.
16. Lehmann U, Celikkaya G, Hasemeier B, Länger F, Kreipe H. Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. *Cancer Res* 2002;62:6634–8.
17. Simpson DJ, Clayton RN, Farrell WE. Preferential loss of death associated protein kinase expression in invasive pituitary tumors is associated with either CpG island methylation or homozygous deletion. *Oncogene* 2002;21:1217–24.
18. Raveh T, Droguett G, Horwitz MS, De Pinho RA, Kimchi A. DAP-kinase activates p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat Cell Biol* 2001;3:1–7.
19. Wang W-J, Kuo J-C, Yao C-C, Chen R-H. DAP-kinase induces apoptosis by suppressing integrin activity and disrupting matrix survival signals. *J Cell Biol* 2002;159:169–79.
20. Tang X, Khuri FR, Lee JJ, et al. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J Natl Cancer Inst (Bethesda)* 2000;92:1511–6.
21. Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* 1999;93:4347–53.